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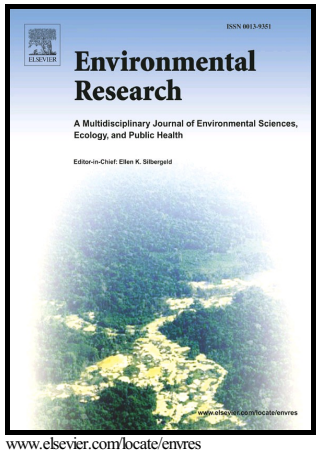

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Higher plasma oxidative damage and lower plasma antioxidant defences in an Arctic seabird exposed to longer perfluoroalkyl acids

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ABSTRACT

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) may cause detrimental effects on physiological function and reproduction of Arctic animals. However, there is a paucity of information on the link between PFASs and oxidative stress, which can have

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potential detrimental effects on key fitness traits, such as cellular homeostasis or reproduction. We have examined the correlations between multiple blood-based markers of oxidative status and several perfluoroalkyl acids (i.e., with 8 or more carbons) in male Arctic black-legged kittiwakes (*Rissa tridactyla*) during the pre-laying period. Higher protein oxidative damage was found in those birds having higher concentrations of perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriA) and perfluorotetradecanoic acid (PFTeA). Lower plasmatic non-enzymatic micro-molecular antioxidants was found in those birds having higher concentrations of perfluoroundecanoic acid (PFUnA), PFDoA and PFTeA. Effect size estimates showed that the significant correlations between PFASs and oxidative status markers were intermediate to strong. The non-enzymatic antioxidant capacity (including antioxidants of protein origin) was significantly lower in those birds having higher plasma concentration of linear perfluorooctanesulfonic acid (PFOSlin). In contrast, the activity of the antioxidant enzyme glutathione peroxidase in erythrocytes was not associated with any PFAS compounds. Our results suggest that increased oxidative stress might be one consequence of long-chain PFAS exposure. Experimental work will be needed to demonstrate whether PFASs cause toxic effects on free-living vertebrates through increased oxidative stress.

Keywords: Antioxidants, Birds, Kittiwake, PFASs, Svalbard

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1. Introduction

Ecotoxicological studies have so far extensively directed their attention toward legacy persistent organic pollutants (POPs) like organochlorine pesticides (OCPs) and polychlorobiphenyls (PCBs) (Stockholm Convention, 2009). In contrast, less attention has been given to the environmental toxicity of other organic contaminants in the Arctic, such as chlorinated paraffins, phthalates, siloxanes or the perfluoroalkyl and polyfluoroalkyl substances (PFASs; AMAP, 2017). Among these, PFASs remain comparatively much less investigated (DeWitt, 2015). PFASs are synthetically manufactured chemicals, produced since the 1950s, that are widely used for numerous industrial and commercial purposes as water repellents and surfactants (e.g., impregnation agents for carpets, papers and textiles, fire-fighting foam, non-stick coating and waterproof clothing) (Kissa, 2001; Jensen and Leffers, 2008). Chemically and thermally stable, PFASs are highly persistent in the environment and have been detected globally in both wildlife and humans (Key et al., 1997; Giesy and Kannan, 2001; Lau et al., 2007; Muir and DeWit, 2010). Because of oceanic currents and atmospheric long-range transport, PFASs and their precursors and breakdown products can reach high latitudes, such as the Arctic Ocean (Giesy and Kannan, 2001; Prevedouros et al., 2006; Butt et al., 2010). Once deposited in the Arctic marine ecosystem, PFASs bio-accumulate in living organisms and bio-magnify along the food webs (Tomy et al., 2004; Kannan et al., 2005; Haukås et al., 2007; Kelly et al., 2009; Fang et al., 2014). Importantly, (i) PFASs have long half-lives, which facilitates their biomagnification through the food webs depending on the species and PFAS congener (Muir and DeWit, 2010); (ii) the long and odd carbon-chain-length PFASs appear to be

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more bio-accumulative and toxic than the short and even-chain length PFASs in wildlife (Martin et al., 2004; Verreault et al., 2005; Conder et al., 2008; Berntsen et al., 2017).

It is worthwhile to note that while PFASs have been produced for over 50 years, it is only since late 1990s that their occurrence in the environment has come under scientific scrutiny. PFASs have raised recent concerns about their potential physiological disrupting properties and negative impacts on reproductive fitness in wildlife (multiple species in Bossi et al., 2005; lesser black-backed gull in Bustnes et al., 2008; northern fulmar in Braune et al., 2011; zebrafish in Liu et al., 2011; tree swallow in Custer et al., 2012; black-legged kittiwake and northern fulmar in Nøst et al., 2012; black-legged kittiwake in Tartu et al., 2014a,b; black-legged kittiwake in Blévin et al., 2017a,b; glaucous gull in Melnes et al., 2017; common eider, black guillemot, black-legged kittiwake, glaucous gull, arctic skua and great skua in Haarr et al., 2018).

Increased molecular oxidative damage and disruption of antioxidant defences are suspected as important mechanisms through which PFASs could be detrimental for cell function and, possibly, for organism health (e.g., Marasco and Costantini, 2016). Experimental evidence on laboratory models found that PFASs may increase production of reactive oxygen species (ROS), increase molecular oxidative damage and up- or down-regulate antioxidant defences (Yao et al., 2005; Eriksen et al., 2010; Liu et al., 2011). Further work found that the perfluoroundecanoic and perfluorododecanoic acids (PFUnA and PFDoA, respectively) are equally potent inducers of stress response genes relative to perfluorooctane sulfonic acid (PFOS) and perfluorononanoic acid (PFNA) and that the effect of carbon-chain-length was more important than the functional group in determining oxidative stress (Nobels et al., 2010). There is thus good reason to expect

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that long-chain PFASs might cause dysregulation of the oxidative homeostasis, leading to accumulation of oxidative damage to key biomolecules like proteins or nucleic acids. However, the effect of PFAS exposure on oxidative stress is almost unknown for wildlife. Nakayama et al. (2008) found changes in antioxidant gene expression in cormorants (*Phalacrocorax carbo*) exposed to PFASs. Sletten et al. (2016) did not find any significant relationship between plasma PFAS concentrations and the activity of the antioxidant enzyme superoxide dismutase in plasma of white-tailed eagle (*Haliaeetus albicilla*) nestlings, while Haarr et al. (2018) did not find any significant relationship between PFASs and amount of DNA damage in lymphocytes in common eider (*Somateria mollissima*), black guillemot (*Cepphus grylle*), black-legged kittiwake (*Rissa tridactyla*), glaucous gull (*Larus hyperboreus*), arctic skua (*Stercorarius parasiticus*), and great skua (*Stercorarius skua*).

Long-lived species, like many polar seabirds that occupy high trophic levels, are exposed to a greater risk of accumulation and sensitivity to high concentrations of contaminants. In Svalbard (European Arctic), a number of studies showed that black-legged kittiwakes (*Rissa tridactyla*, hereafter "kittiwake") are chronically exposed to a complex cocktail of organic contaminants and trace elements, which are known to correlate with physiological metrics, impaired individual fitness and population dynamics (Tartu et al., 2013, 2014a,b; Goutte et al., 2015; Blévin et al., 2016, 2017a,b). It is, however, unknown whether exposure of kittiwakes to PFASs is associated with markers of oxidative damage and antioxidant protection.

In this study, we have examined the correlations between blood-based markers of oxidative status and several PFAS compounds in adult male kittiwakes during the pre-laying period, while controlling for a number of potential confounding factors that

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might affect the oxidative status independently from PFASs (i.e., body condition, body size, both time and day of blood sampling, hormonal status; reviewed in Costantini, 2014). As with the hormonal status, we measured plasma levels of testosterone, baseline corticosterone and luteinizing hormone because prior work found large individual variation among kittiwakes (Tartu et al., 2013, 2014a,b) and significant effects on organism's oxidative status (Costantini, 2014), which could affect the relationships between PFAS and oxidative status markers. We focused on males because this investigation on oxidative stress is part of a larger project aiming at assessing the overall consequences (ornament coloration, fecundity, oxidative stress, sexual hormones) of PFASs exposure in males during the pre-laying stage (nest site defence, pair-bonding, copulation, nest building) a period during which males appear to be sensitive to pollutants (Tartu et al., 2013).

We have also examined whether the effect size of the association between each oxidative status marker and each PFAS compound varies according to their carbon-chain-length (C_{8-14}) because the toxicity of PFASs may increase with carbon-chain-length.

2. Materials and methods

2.1 Sampling

Fieldwork was conducted in 2016 on a colony of Arctic kittiwakes at Kongsfjord (78° 54' N; 12° 13' E), Svalbard. Blood samples were collected from 50 adult males during the pre-laying period (courtship and mating period), from 25th May to 6th June. Birds were caught on their nest with a loop at the end of a long pole. Within 3 minutes since capture 0.5 ml of blood were taken from the brachial vein using a heparinized syringe

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and a 25-gauge needle. This blood sample was used to measure oxidative status markers and hormones. Straightaway, a second sample of venous blood (ca. 2 ml) was collected using another syringe and this blood sample was used to assess the PFASs burden. We collected blood in two separate phases because the first one should have been collected within 3 minutes from capture in order to obtain a correct value of basal corticosterone and, given that analyses of PFASs require a large volume of blood, a second bleeding was necessary. Then, tarsus, skull (head + bill) and wing length were measured using a calliper (nearest 0.1 mm) and body mass was taken using a Pesola spring balance (nearest 5 grams). Blood samples were stored on ice in the field. On average, blood samples were stored on ice for 3h40min (min: 1h30min; max 9h55min) before being centrifuged and stored at -80°C. Plasma and red blood cells, obtained after centrifugation, were kept frozen separately, either at -80°C for subsequent oxidative status markers or at -20°C for PFAS analyses. All samples were analysed within 4 months since collection.

2.2 Hormone assays

Plasma levels of testosterone, baseline corticosterone and luteinizing hormone (LH) were measured by radioimmunoassay at the Centre d'Etudes Biologiques de Chizé following protocols previously validated (e.g., Jouventin and Mauget, 1996; Tanvez et al., 2004; Lormée et al., 2013). Briefly, testosterone and corticosterone were extracted using diethyl ether and ethyl ether, respectively. Plasma concentrations of all three hormones were measured by radioimmunoassay. As with corticosterone, a commercial antiserum against corticosterone-3-(O-carboxy-methyl) oxime bovine serum albumin conjugate (Biogenesis, UK) was used. The lowest detectable quantities significantly

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different from zero at a 90% confidence level were 0.05 ng/ml for testosterone, 0.4 ng/ml for corticosterone and 1.7 ng/ml for LH. All samples were analysed in duplicate. Corticosterone and LH were analysed in a single run and the mean coefficient of variation was 4.9 and 12.0%, respectively. Testosterone was analysed in two runs and the coefficients of variation were 11.2 and 11.5%.

2.3 Oxidative status markers

One marker of plasma oxidative damage (protein carbonyls), one marker of plasma non-enzymatic antioxidant capacity and one red blood cell antioxidant enzyme (glutathione peroxidase) were measured at the Centre d'Etudes Biologiques de Chizé using standard methods (e.g., Costantini et al., 2013, 2017). Protein carbonyls (marker of oxidative protein damage) were measured using the Protein Carbonyl Colorimetric assay (Cayman Chemical Company, Ann Arbor, USA). This assay is based on the colorimetric method proposed by Levine et al. (1990). A same volume of plasma was used for all samples and the amount of carbonyls was standardised by the plasma protein concentration according to manufacturer's instructions. Protein carbonyls were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH). The absorbance was read at 370 nm. The extinction coefficient for DNPH (0.022/µM/cm) was used to calculate the concentration of protein carbonyls, which was expressed as nmol/mg protein (amount of carbonyls generated per unit of protein) or as total nmol obtained by multiplying the concentration of carbonyls by the concentration of plasma proteins (i.e., total amount of carbonyls in the sample, which is also dependent on the amount of substrates available, i.e., proteins). The mean coefficient of variation of duplicates was 11.5%. The metric expressed as nmol/mg indicates the

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amount of carbonyls that occurs in a same amount of protein, thus this is standardised by the amount of substrates (i.e., proteins) that can be carbonylated. The second metric expressed as total amount of carbonyls indicates the total amount of carbonyls that occurs in the tissue, which is influenced by the amount of proteins available. This second metric is also important because accumulation of carbonyls is detrimental for the cells (Halliwell and Gutteridge, 2015). The OXY-Adsorbent test (Diacron International, Italy) was used to quantify the non-enzymatic antioxidant capacity of plasma against HOCl. Values were expressed as either mM of HOCl neutralised or as mM of HOCl neutralised/mg protein to estimate the antioxidant potential of micromolecular antioxidants (e.g., vitamins, carotenoids, glutathione) without the contribution of proteins (i.e., non-enzymatic micro-molecular antioxidant capacity). The correlation between OXY values and protein concentration was actually high and significant ($r = 0.76$, $p < 0.001$), which is to be expected because plasma proteins, such as albumin, are prone to react with HOCl. Although free-radical trapping properties vary among proteins, standardising OXY values by the concentration of total plasma proteins gave us some kind of control about the contribution of proteins to OXY. The mean coefficient of variation of duplicates was 9.4%. The Ransel assay (RANDOX Laboratories, UK) was used to measure the activity of the antioxidant enzyme glutathione peroxidase (GPX) in haemolysates (red blood cells diluted with distilled water). Values were expressed as Units of GPX/mg of protein of haemolysate. The mean coefficient of variation of duplicates was 8.1%. The Bradford protein assay (Bio-Rad Laboratories, Hercules, USA) with bovine albumin as a reference standard was used to measure the concentration of proteins in both plasma samples and haemolysates.

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2.4 PFAS analyses

Perfluoroalkyl acids (sulfonic and carboxylic) were analysed in plasma at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. The following compounds were analysed in each plasma sample. Sulfonic: perfluoropropanesulfonic acid (PFPS), perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS), perfluoroheptanesulfonic acid (PFHpS), perfluorooctanesulfonamide (PFOSA), perfluorooctane sulfonic acid (PFOSlin), branched perfluorooctane sulfonic acid (PFOSbr), perfluorononane sulfonic acid (PFNS), perfluorodecane sulfonic acid (PFDcS); Carboxylic: perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoate (PFDcA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriA), perfluorotetradecanoic acid (PFTeA) and two precursor compounds, the fluorotelomer sulfonates (6:2 FTS and 8:2 FTS). PFASs with concentrations below the limit of quantification (LOQ) were replaced with a value equal to (LOQ × detection frequency) when the detection frequency (percentage of detection) was > 50% (e.g., James et al., 2002). A 0.2 ml aliquot of plasma spiked with internal standards (carbon labeled PFAS) was extracted in methanol (1 ml) by repeated sonication and vortexing. The supernatant was cleaned-up using ENVICarb graphitized carbon absorbent and glacial acetic acid. Extracts were analysed by UPLC/MS/MS. Recovery of the internal standards ranged between 86.3% and 120%. Results were validated with blanks (clean and empty glass tubes treated like a sample) and standard reference material (SRM; 1957 human serum from NIST) run with every 10 samples. The deviation of the target concentrations in the SRM were within the laboratory's accepted range (69 - 119%). Blanks varied between

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concentrations below the instrument detection limits and 30 pg/g and were applied as the LOD in the form of 3 times the average concentration.

2.5 Body size and body condition

A body size index was estimated using the PC1 from a PCA of tarsus, skull and wing length (loadings, 0.73, 0.83 and 0.45, respectively). As far as the body condition, we did not use the ratio of body mass onto body size nor the residuals of a linear regression of body mass onto body size because both indices have been criticized (García-Berthou, 2001). Rather we included both body mass and body size index as factors in the models. In doing so, the coefficient estimate of body mass is calculated considering the effect of body size, thus the outcome reflects the effect of body condition on the given marker of oxidative status (García-Berthou, 2001).

2.6 Statistical analyses

Generalized linear models were performed using the software STATISTICA 10 (StatSoft. Inc., Tulsa, OK, USA) to assess relationships between each oxidative status marker (protein carbonylation, non-enzymatic antioxidant capacity, non-enzymatic micro-molecular antioxidant capacity and glutathione peroxidase) and the following predictor variables: PFASs congener (PFOSlin, PFNA, PFDcA, PFUnA, PFDoA, PFTriA or PFTeA), body size index, body mass, hormonal status, day of blood sampling and time of blood sampling. Correlations among PFASs were very variable (from small 0.32 to high 0.92), with smaller correlations between PFASs of different chain length and stronger correlations between PFASs of similar chain length. Thus, we did not rely on Principal Components Analysis because it would not allow us to (i)

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capture all the information associated with single congeners and (ii) test whether connections between PFASs and oxidative status markers were dependent on carbon chain length. All predictor variables were scaled to mean of 0 and standard deviation of 1. These predictor variables were included in all models because prior work showed that each of them can be significantly associated with oxidative status markers (reviewed in Costantini, 2014). The two metrics of protein oxidative damage (protein carbonyls per mg of protein and total protein carbonyls) were combined using the PC1 from a Principal Components Analysis (PCA) because they were highly correlated ($r = 0.79$, $p < 0.001$). This was not done for the non-enzymatic capacity of plasma because both variables were not correlated ($r = 0.22$, $p = 0.13$). The hormonal status was estimated using the PC1 from a PCA of corticosterone, testosterone and luteinizing hormone (loadings, 0.39, 0.61 and 0.84, respectively). A normal error function and an identity-link function were applied to models of non-enzymatic antioxidant capacity and GPX. A gamma error function and an identity-link function were applied to models of protein carbonyls and non-enzymatic micro-molecular antioxidant capacity. These functions were selected because the model had the best fitting to the dataset according to the Akaike Information Criterion. Preliminary analyses showed that the time elapsed from the collection of blood to its storage was not significantly correlated (p-value > 0.45) with any marker of oxidative status nor hormones, thus it was not further considered in the analyses. Visual inspection of residuals, Q-Q plots and Cook's distance did not highlight any violation of normality and homogeneity of variance nor the presence of outliers (all samples were below a 0.5 Cook's distance). The variance inflation factor was always below 2, indicating that multicollinearity was low. The multicollinearity is thought to be high and problematic when the variance inflation factor is higher than 5.

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The "compute.es package" (Del Re, 2013) in R (R Core Team et al., 2013) was used to calculate the standardized effect size Hedges' g from test statistics of oxidative status markers that had significant associations with PFASs (i.e., protein oxidative damage and non-enzymatic micro-molecular antioxidant capacity). The "forestplots function" of the "metafor package" in R was used to visualise boxplots of effect size and 95% confidence interval. Effect sizes were considered to be small (Hedges' g = 0.2, explaining 1% of the variance), intermediate (g = 0.5, explaining 9% of the variance) or large (g = 0.8, explaining 25% of the variance) according to Cohen (1988).

3. Results

Concentrations of detectable PFASs are reported in Table 1 together with other variables measured in kittiwakes. Six out of 20 PFASs (i.e. PFOSlin, PFNA, PFDcA, PFUnA, PFDoA, PFTriA) were detectable in all individuals, while one PFAS (i.e. PFTeA) was detectable in 33 out of 50 individuals. PFOSlin, PFUnA and PFTriA concentrations were the highest of all PFASs measured in the investigated samples, with a percentage contribution for each kittiwake ranging from 23.4 to 54.5%, from 20.3 to 34.8% and from 11.8 to 35.2%, respectively. The percentage contribution of all other detected PFASs ranged between 0.1 and 10.6%. PFOSA, PFBS, PFPS, PFHxS, PFHpS, PFOSbr, PFNS, PFDcS, PFHxA, PFHpA, PFOA, and the two precursor fluorotelomer sulfonates (6:2 FTS and 8:2 FTS) were below the detection limit in all the investigated samples.

Protein oxidative damage was significantly higher in those birds having higher plasma concentration of PFDoA, PFTriA or PFTeA (Table 2). Effect size estimates increased with chain length of PFASs (indicating an increase of protein damage with

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chain length) and were significantly different from zero) for PFDoA (95% confidence interval: 0.06 to 1.25), PFTriA (95% confidence interval: 0.01 to 1.20) and PFTeA (95% confidence interval: 0.29 to 1.54; Fig. 1). The non-enzymatic micro-molecular antioxidant capacity was significantly lower in those birds having higher plasma concentration of PFUnA, PFDoA or PFTeA (Table 2). There was also a near-significance tendency of the non-enzymatic micro-molecular antioxidant capacity to be lower in birds with higher plasma PFTriA (Table 2). Effect size estimates were larger for longer PFASs (indicating a decrease of micro-molecular antioxidants with chain length) and were significantly different from zero for PFUnA (95% confidence interval: -1.20 to -0.02), PFDoA (95% confidence interval: -1.35 to -0.14) and PFTeA (95% confidence interval: -1.23 to -0.04; Fig. 2). The non-enzymatic antioxidant capacity including the contribution of antioxidant of protein origin was significantly lower in those birds having higher plasma concentration of PFOSlin, but it was not associated with any other PFAS congener (Table 2). The activity of GPX was not associated with any PFAS compounds (Table 2).

Finally, our models showed that (i) kittiwakes in poorer body condition had more plasma protein carbonyls, (ii) the non-enzymatic antioxidant capacity was higher in kittiwakes sampled later in the day, and (iii) the non-enzymatic micro-molecular antioxidant capacity was higher in kittiwakes having lower concentrations of hormones.

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4. Discussion

Our results provide the first evidence in wild vertebrates that the correlation between oxidative status markers and PFASs is stronger for long-chain congeners. We found that male kittiwakes having higher plasma concentrations of long-chain PFASs had more

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protein oxidative damage and less plasma antioxidants after controlling statistically for potentially confounding variables. The non-enzymatic antioxidant capacity (including antioxidants of protein origin) was significantly lower in kittiwakes having higher plasma concentration of PFOSlin. On the other hand, the activity of glutathione peroxidase in erythrocytes was not related to any PFAS congener. Effect size estimates were intermediate to large, indicating that PFASs explained from 9 to more than 25% of the variance in protein oxidative damage and non-enzymatic micro-molecular antioxidant capacity of plasma (Cohen, 1988). Intermediate effect sizes are suggested to be biologically meaningful because average proportions of variance explained in ecological, evolutionary and physiological studies is usually below 7% (Møller and Jennions, 2002). Our effect size estimates were also larger than those found in the comparison of oxidative status markers between animals living in polluted (e.g., air pollution, heavy metals) and unpolluted sites (Isaksson, 2010).

PFOSlin, PFUnA and PFTriA concentrations were the highest of all PFASs measured in the investigated samples, with a percentage contribution for each kittiwake ranging from 11.8 to 54.5%. The concentration of PFOSlin was higher than that previously recorded in males from the same kittiwake population in 2012 (13.4 vs. 10.6 pg/g ww in Blévin et al., 2017a). In contrast, the average concentrations of PFUnA (10.3 vs. 12.1 pg/g ww in Blévin et al., 2017a) and of PFTriA (8.6 vs. 11.6 pg/g ww in Blévin et al., 2017a) were both lower in our study than in prior work (Blévin et al., 2017a). One reason for such differences might be because Blévin et al. (2017a) measured PFASs of male kittiwakes caught during the chick rearing phase. Work on male glaucous gulls during the incubation period in Svalbard found levels of PFOSlin

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similar to ours, while those of PFUnA (4.4 pg/g ww) and of PFTriA (3.9 pg/g ww) were much lower than those we recorded (Melnes et al., 2017).

The strength of the correlation between oxidative status markers and PFASs increased with the chain length of the congener. While persistent in the environment, PFASs with fewer than eight carbons, such as PFHxA, and PFSAs with fewer than six carbons, such as PFBS, are generally less bioaccumulative in wildlife and humans. However, it is still unclear whether chain length affects toxicity. For example, Vongphachan et al. (2011) experimentally found that SC-PFASs altered the expression of TH-responsive genes in chicken embryonic neuronal cells to a greater extent than LC-PFASs. On the other hand, exposure of laboratory animals to long-chain congeners produces detrimental reproductive, developmental, and systemic effects (Lau et al., 2007; Conder et al., 2008; Jensen and Leffers, 2008; Concawe, 2016) and the toxic effects of PFASs on rat brain cells increased with increasing carbon chain length (Berntsen et al., 2017). Interestingly, the toxic effects of PFASs were attenuated by the antioxidant vitamin E, indicating a possible involvement of oxidative stress in the reduction of cell viability (Concawe, 2016). Further work showed that, compared to short-chain PFASs, long-chain PFASs are stronger inducers of the response of genes regulating the cell oxidative status (Nobels et al., 2010), suggesting that they might be able of causing a stronger oxidative insult to the cells, which need to upregulate their antioxidant response.

Effects of PFAS exposure on oxidative status have been moderately investigated in laboratory models and almost unexplored in wild animals. Thus, limited information is available for a comparison with our results and interpretation. Protein carbonylation arises from overproduction of ROS by metabolic reactions that use oxygen and shift the

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balance between oxidant/antioxidant statuses in favour of the oxidants (Halliwell and Gutteridge, 2015). Protein carbonylation also occurs when carbonyls are introduced into proteins through the reactions with lipid oxidative damage products (malondialdehyde and hydroxynonenal; Halliwell and Gutteridge, 2015). Carbonylation is mostly irreversible and results in alteration of protein structure and function. Only a small fraction of carbonylated proteins can be removed through proteasome-dependent proteolysis. Work carried out on laboratory models found evidence that exposure to PFASs may change expression of genes regulating proteasome activation and proteolysis (Lau et al., 2007; Nilsen et al., 2008; Zhang et al., 2012). It is, however, unclear, whether such changes in gene expression make proteins one important target of the pro-oxidant effects of PFASs. This is important because when protein carbonyls accumulate, they tend to aggregate leading to cell death, tissue injury and development of disorders. Several studies found higher amounts of plasma protein carbonyls in individuals affected by a given disease (Winterbourn et al., 2003; Hlaváčková et al., 2017), suggesting a potential role of protein carbonylation in disease progression.

Depletion of circulating non-enzymatic antioxidants might reflect increased oxidation due to reaction with ROS, reduced intake from diet or mobilisation of antioxidants from blood to other target tissues. Irrespective of the reason, prior work on other bird species found evidence that circulating antioxidants may be linked to important individual or population fitness-related traits. For example, Saino et al. (2011) found that barn swallows (*Hirundo rustica*) with lower plasma non-enzymatic antioxidants had reduced probability of survival. Beaulieu et al. (2013) found that Gentoo (*Pygoscelis papua*) and Adélie (*Pygoscelis adeliae*) penguins from increasing

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populations had higher plasma non-enzymatic antioxidant capacity than those from decreasing populations.

Metabolic activity is one important source of ROS production. Thus the association we found between PFASs and oxidative status markers might mirror an effect of PFASs on metabolism. Prior work on the same kittiwake population found a positive association between the long-chain PFTriA and resting metabolic rate in females but not in males (Blévin et al., 2017b). Thus, the association between PFASs and oxidative status markers does not appear, at least for males, to be due to a dysregulation of general body metabolism. The lack of an effect on metabolic rate does not reject the hypothesis that ROS production might have been higher in the more contaminated birds. For example, PFASs might have localised effects on important ROS generators (e.g., mitochondria of red blood cells or of other target tissues) without compromising the metabolism of the whole organism. *In vitro* studies found that PFAS exposure may impair mitochondrial activity and lead to increased rates of reactive oxygen species production (O'Brien and Wallace, 2004).

It is unclear why the activity of glutathione peroxidase was not associated with any PFAS compound. It might be that up-regulation of this enzyme might have been too costly for the birds given the imminent start of breeding activity or that any effects of PFASs on oxidative status did not come through the pathways involving glutathione peroxidase. The biochemical function of glutathione peroxidase is to reduce hydrogen peroxide to water and organic hydroperoxides to their corresponding alcohols (Halliwell and Gutteridge, 2015). Thus, we cannot exclude that results would have been different if another antioxidant enzyme with a different biochemical function would have been measured. There are, however, many discrepancies in the literature about the response

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of antioxidant enzymes to PFAS exposure. For example, prior work did not find any association between PFASs exposure and whole-body catalase activity in the planktonic crustacean *Daphnia magna* (Li, 2010) or plasma superoxide dismutase activity in white-tailed eagle (*Haliaeetus albicilla*) nestlings (Sletten et al., 2016). In contrast, exposure to PFASs caused induction of antioxidant enzymes in response to oxidative stress and the suppression of molecular chaperones, leading to reduction in protein stability, in cormorants (Nakayama et al., 2008), affected catalase activity in hepatocytes of freshwater tilapia *Oreochromis niloticus* (Liu et al., 2011), expression of antioxidant genes Sod1, Sod2, Gpx2 and Nqo1 in mouse pancreas (Kamendulis et al., 2014). Irrespective of the mechanisms involved, the activity of GPX in erythrocytes does not appear to be an informative marker about the impact on oxidative status of the PFASs we have measured in this work.

In conclusion, our work shows that higher protein oxidative damage was found in those birds having higher concentrations of PFDoA, PFTriA and PFTeA. Lower plasmatic non-enzymatic micro-molecular antioxidants was found in those birds having higher concentrations of PFUnA, PFDoA and PFTeA. Experimental work will be needed to ascertain whether the correlation between individual PFAS burden and oxidative status markers reflects a direct toxic effect of PFASs on oxidative homeostasis. It will also be important to determine whether increased oxidative damage or decreased antioxidant defences turn into a reduction in survival probability or lifetime reproductive success. Resistance against oxidative stress may also decrease with chronological age (Marasco et al., 2017). Thus, we highlight the importance of assessing in future studies whether the effects of PFASs on oxidative status markers are stronger in older individuals.

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Conflict of interest

The authors declare no competing financial interests.

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Table 1. Descriptive statistics of all perfluoroalkyl substances, oxidative status markers (i.e., responde variables) and several individual metrics (potential confounding factors) measured in 50 male black-legged kittiwakes from Svalbard.

Variable	**Mean ± Standard deviation**
CONTAMINANT	
PFOSlin - Linear perfluorooctane sulfonic acid (ng/g ww)	13.4 ± 6.2
PFNA - Perfluorononanoic acid (ng/g ww)	2.0 ± 0.9
PFDcA - Perfluorodecanoic acid (ng/g ww)	2.9 ± 1.2
PFUnA - Perfluoroundecanoic acid (ng/g ww)	10.3 ± 3.7
PFDoA - Perfluorododecanoic acid (ng/g ww)	1.7 ± 0.8
PFTriA - Perfluorotridecanoic acid (ng/g ww)	8.6 ± 3.1
PFTeA - Perfluorotetradecanoic acid (ng/g ww)	1.0 ± 0.8
OXIDATIVE STATUS MARKERS – RESPONSE VARIABLES	
Protein carbonyls (nmol/mg protein)	1.8 ± 0.6
Total protein carbonyls (nmol)	22.5 ± 8.2
Glutathione peroxidase (Units/mg protein)	0.4 ± 0.2
Non-enzymatic antioxidant capacity (mM HOCl neutralised)	169.9 ± 37.1
Non-enzymatic micro-molecular antioxidant capacity (mM HOCl neutralised/mg protein)	13.0 ± 2.0
INDIVIDUAL TRAITS	
Body mass (g)	416.2 ± 23.1
Skull length (mm)	93.9 ± 1.6
Tarsus length (mm)	34.9 ± 1.0
Wing length (mm)	317.1 ± 6.3
Baseline corticosterone (ng/ml)	9.6 ± 7.4
Testosterone (ng/ml)	1.7 ± 1.4
Luteinizing hormone (ng/ml)	6.2 ± 2.4

Table 2. The table shows the outcomes of generalized linear models performed to test the effect of each PFASs congener on oxidative status markers. Each model also included a number of potential confounding factors that may affect oxidative status markers independently from PFASs. Significant effects are shown in bold type. Note that the coefficient estimate for the relationship between body mass and the dependent variable (i.e., oxidative status marker) is calculated considering the effect of body size, thus it actually indicates the covariation between a given marker and the individual body condition (García-Berthou, 2011). GPX = glutathione peroxidase; N-E Antioxs = non-enzymatic antioxidant capacity of plasma; N-E Micromol Antioxs = non-enzymatic micro-molecular antioxidant capacity of plasma; ce ± se = coefficient estimate ± standard error.

	Protein Oxidative Damage		**GPX**		**N-E Antioxs**		**N-E Micromol Antioxs**	
Main effect included in the model	ce ± se	P	ce ± se	P	ce ± se	P	ce ± se	p
PFOSlin	0.03±0.12	0.817	0.02±0.02	0.462	-13.7±5.0	**0.006**	-0.20±0.28	0.484
sampling date	0.11±0.13	0.406	0.03±0.03	0.309	-16.6±5.0	**0.001**	0.18±0.31	0.546
sampling time	-0.20±0.11	0.074	0.03±0.02	0.196	14.3±4.6	**0.002**	0.22±0.28	0.425
body mass	-0.29±0.12	**0.013**	-0.02±0.03	0.407	4.0±5.0	0.424	0.00±0.29	1.000
body size	-0.02±0.11	0.840	0.02±0.02	0.403	-5.5±4.7	0.236	0.10±0.27	0.704
hormonal status	-0.10±0.11	0.391	0.02±0.02	0.348	4.0±4.7	0.391	-0.94±0.28	**0.001**
PFNA	-0.03±0.13	0.852	0.01±0.03	0.764	-1.0±6.0	0.872	-0.09±0.34	0.804
sampling date	0.08±0.15	0.580	0.02±0.03	0.433	-10.7±6.0	0.077	0.22±0.35	0.534

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sampling time	-0.20±0.11	0.074	0.03±0.02	0.208	14.2±5.0	**0.004**	0.22±0.28	0.423
body mass	-0.30±0.12	**0.011**	-0.02±0.03	0.395	5.2±5.4	0.336	0.01±0.29	0.977
body size	-0.01±0.11	0.896	0.02±0.02	0.353	-7.4±5.0	0.137	0.08±0.27	0.768
hormonal status	-0.08±0.11	0.460	0.03±0.02	0.262	0.28±4.9	0.953	-0.98±0.27	**<0.001**
PFDcA	0.22±0.13	0.106	0.00±0.03	0.904	-0.65±5.8	0.910	-0.49±0.31	0.116
sampling date	0.22±0.14	0.104	0.02±0.03	0.493	-10.5±5.0	0.075	-0.02±0.33	0.950
sampling time	-0.18±0.11	0.088	0.03±0.02	0.195	14.1±4.9	**0.004**	0.21±0.27	0.438
body mass	-0.22±0.12	0.056	-0.02±0.03	0.407	5.1±5.6	0.357	-0.07±0.29	0.809
body size	-0.03±0.11	0.795	0.02±0.02	0.352	-7.4±5.0	0.140	0.09±0.27	0.723
hormonal status	-0.14±0.11	0.204	0.03±0.02	0.249	0.24±4.9	0.960	-0.92±0.27	**0.001**
PFUnA	0.14±0.13	0.268	0.00±0.03	0.970	-3.3±5.6	0.562	-0.62±0.29	**0.035**
sampling date	0.19±0.14	0.181	0.02±0.03	0.517	-12.0±5.8	**0.038**	-0.07±0.32	0.822
sampling time	-0.18±0.11	0.098	0.03±0.02	0.196	13.9±4.9	**0.005**	0.19±0.27	0.483
body mass	-0.24±0.12	**0.045**	-0.02±0.03	0.403	4.2±5.7	0.453	-0.13±0.29	0.659
body size	-0.03±0.11	0.807	0.02±0.02	0.348	-7.1±5.0	0.154	0.12±0.26	0.644
hormonal status	-0.13±0.11	0.237	0.03±0.02	0.247	0.86±4.9	0.861	-0.87±0.26	**0.001**
PFDoA	0.29±0.13	**0.024**	-0.01±0.03	0.761	-1.5±5.4	0.779	-0.73±0.29	**0.012**
sampling date	0.26±0.14	0.066	0.01±0.03	0.625	-10.9±5.6	0.052	-0.10±0.30	0.736
sampling time	-0.18±0.10	0.088	0.03±0.02	0.199	14.1±4.9	**0.004**	0.21±0.26	0.421
body mass	-0.20±0.11	0.076	-0.02±0.03	0.343	4.9±5.6	0.378	-0.16±0.28	0.563
body size	-0.02±0.11	0.833	0.02±0.02	0.325	-7.2±5.0	0.148	0.14±0.25	0.574
hormonal status	-0.14±0.10	0.176	0.03±0.02	0.220	0.21±4.8	0.965	-0.95±0.25	**<0.001**
PFTriA	0.30±0.14	**0.036**	0.00±0.03	0.923	2.0±5.9	0.735	-0.58±0.32	0.064
sampling date	0.26±0.15	0.075	0.02±0.03	0.597	-8.8±6.1	0.153	-0.12±0.34	0.725
sampling time	-0.18±0.11	0.094	0.03±0.02	0.214	14.5±5.1	**0.004**	0.10±0.27	0.725
body mass	-0.26±0.11	**0.026**	-0.02±0.03	0.368	5.7±5.5	0.299	-0.08±0.28	0.763
body size	-0.06±0.12	0.632	0.02±0.02	0.348	-7.9±5.2	0.124	0.20±0.27	0.454
hormonal status	-0.14±0.11	0.181	0.03±0.02	0.226	-0.13±4.9	0.979	-0.92±0.26	**<0.001**
PFTeA	0.33±0.11	**0.003**	-0.02±0.03	0.482	7.4±5.4	0.171	-0.65±0.29	**0.028**
sampling date	0.22±0.12	0.070	0.01±0.02	0.664	-7.4±5.1	0.145	0.05±0.29	0.852
sampling time	-0.27±0.11	**0.013**	0.03±0.02	0.149	12.3±5.0	**0.015**	0.40±0.27	0.144
body mass	-0.25±0.11	**0.018**	-0.03±0.03	0.317	6.4±5.3	0.227	-0.05±0.28	0.858
body size	-0.05±0.11	0.668	0.03±0.02	0.275	-9.0±5.0	0.072	0.19±0.26	0.461
hormonal status	-0.19±0.10	0.062	0.03±0.02	0.171	-1.9±4.9	0.704	-0.84±0.27	**0.001**

Figure captions

Figure 1. Mean estimates of effect size and 95% confidence interval are shown. These were calculated from the statistical outcomes showing the effect of each PFAS congener on protein oxidative damage. Estimates are positive when values of damage are higher in birds having higher plasma concentration of a given PFAS congener. Note that effect size estimates are significant when the 95% confidence interval does not include zero. Numbers in bracket indicate carbon-chain-length of each PFAS congener.

Figure 2. Mean estimates of effect size and 95% confidence interval were calculated from the statistical outcomes showing the effect of each PFAS congener on plasma non-enzymatic micromolecular antioxidant capacity. Estimates are negative when values of antioxidants are lower in birds having higher plasma concentration of a given PFAS

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congener. Note that effect size estimates are significant when the 95% confidence interval does not include zero. Numbers in bracket indicate carbon-chain-length of each PFAS congener.

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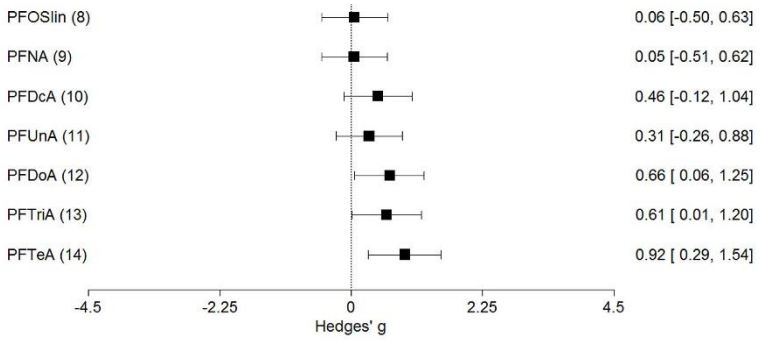

Figure 1

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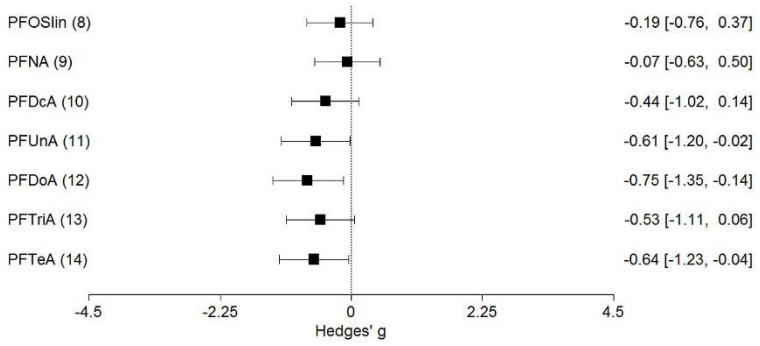

Figure 2

Highlights

- We measured oxidative status markers and PFASs in kittiwakes breeding in Svalbard.
- The association between oxidative damage and PFASs was stronger for longer congeners.
- The association between antioxidants and PFASs was stronger for longer congeners.
- Effect size estimates of significant results were intermediate to strong.
- Increased oxidative stress might be one consequence of long-chain PFAS exposure.

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